Further Researches on the Extrusion of Granules by Trypanosomes and on their Further Development.

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[PLATES 9–11.]

Introduction.

In March, 1911, in the course of some work on trypanosomes carried out at the Wellcome Tropical Research Laboratories, Khartoum, the extrusion of certain granules from trypanosomes was observed by one of us (W. B. F.). The Director of the Laboratories, Dr. Andrew Balfour, was informed of these observations, and he himself shortly after observed a somewhat similar extrusion of granules from Spirochætes (spirochætosis of fowls), an account of which he published.*

In June, 1911, a preliminary note on the subject was communicated to the Royal Society by one of us (W. B. F.). Since then, a great deal of work has been done on the subject by us conjointly, but for the most part independently; by one of us (W. B. F.) at Khartoum and in London, by the other (H. S. R.) at Yei in the Lado Enclave.

As will be seen, the results recorded in the course of this paper go far to confirm the conclusions arrived at in the preliminary note, i.e. that the phenomenon is one connected with a stage in the life-history of the parasite, especially in chronic trypanosomiasis, in which it is found that the trypanosomes disappear from the blood of an affected animal for considerable periods.

These observations offer, too, an explanation of the infectivity of fluids, blood for example, which, while showing absolutely no trace of trypanosomes, will infect susceptible animals, a fact that all workers on trypanosomiasis are acquainted with; they also throw light on that condition which has been spoken of as "a possible ultra-microscopic stage" in these diseases.

Methods.

In the earlier part of these investigations two methods were principally used, they were :—

* 'Brit. Med. Journ.,' April 1, 1911.

(1) Dark-ground illumination used in the ordinary way, but with the addition of a practically monochromatic light, which improved the definition.

(2) A method of "vital staining," the stain used being 0·75-per-cent. toluidin blue in physiological salt solution. This was mixed with blood, gland juice or other fluid to be examined, in a capillary pipette, blown on to a slide, covered with a cover-slip, and ringed with vaseline. With solid organs an emulsion in salt solution was used. The proportion of stain varied with the material from 1–3 to 1–8, according to the rate at which it was felt desirable to cause the staining to take place.

New methods of fixation and staining have also been used; these form the subject of a note appended to this paper. Both of these processes give practically the same results.

I. *On Granules in General in Trypanosomes.*

Besides the nucleus and blepharoplast there are other bodies in many trypanosomes which may, in the ordinary acceptation of the term, be called "granules."

There are certainly two classes of granules to be seen in trypanosomes: (1) those with which we are concerned—probably of nuclear origin and of infective nature; and (2) others which probably represent stored food material. The latter are of importance for us only because of the possibility of their being confused with the former, and here it may be stated that it has been found possible to fix and stain preparations so as to show a difference between them in staining reaction. Further evidence in favour of this differentiation was met with as a side issue in the course of experiments with hypertonic and hypotonic salt solutions, to be described later. It was found that when trypanosomes swelled up under the influence of these solutions many granules disappeared, leaving evident only from one to three. The inference seems to be that the granules which disappeared, owing to alteration in osmotic conditions, are of a quite different nature.

In this paper the word "granule" connotes those first mentioned, whilst the food granules are ignored in our descriptions, unless specifically mentioned.

The following varieties of trypanosomes have been available for study, and granules have been observed in all:—

(1) *T. gambiense* (Sudan), (2) *T. rhodesiense*, (3) *T. brucei*, (4) *T. evansi* (Sudan), (5) *T. nanum* (Sudan), (6) *T. pecaudi* (Sudan), (7) *T. lewisi.*

In all cases the granule, as seen by dark-ground illumination, is a small, sharply defined, highly refractile body, and on vital staining it takes up the toluidin blue rapidly, and shows as a deeply stained, more or less circular body, which contrasts with the lighter tint of the trypanosome body.

The number of granules apparently varies in different species of trypanosomes. They may also vary in size and number in the same species. *e.g.* a strain of *T. nanum*, obtained from cattle, was carried on by passage through gerbils. For two and a half months many of the trypanosomes contained a single large granule. At the end of this period the granule became multiple, and three or four could be seen ; at the same time there was a great diminution in their size. It was noted that, coincidently with this increase in number of granules, the virulence of the strain became greater. We have found that granules are not necessarily always present in trypanosomes. At present we can only generally indicate the stage at which granules may develop, and are unable to say what conditions determine their appearance ; but the following details are the result of our observations :—

T. brucei was investigated in gerbils, in which the disease was fatal in six weeks, and during its course they showed at least two or three exacerbations with a large number of trypanosomes in the blood, with corresponding latent periods when they were absent.

When trypanosomes first appeared in the blood, whether at the beginning of the infection or after a latent period, it was observed that they did not contain granules ; the latter developed about the fourth day after trypanosomes were first seen, and increased in size and number. For about 24 hours trypanosomes with granules were numerous. After this period, when free granules were numerous in the blood, the proportion of trypanosomes containing granules steadily diminished, till finally, though an enormous number of trypanosomes might be present, granules could not be found in any of them. This condition usually preceded a latent phase, or the death of the animal.

We have thus a definite sequence of events during an exacerbation of the disease :—

(1) Trypanosomes without granules.

(2) Trypanosomes showing granules which gradually become larger and very evident.

(3) Many free granules.

(4) Many trypanosomes but no contained granules.

(5) Trypanolytic crisis, or death of the animal.

This was also found to hold good with *T. nanum* and *T. evansi* (Sudan).

In the case of a goat inoculated with *T. brucei*, which lived for 133 days, and whose blood from the end of the first fortnight was always infective, no trypanosomes were at any time discoverable in the blood, which was examined daily for the first two months of the illness. In all the specimens

of blood examined granules have been found.   Similarly in the case of guinea-pigs and rabbits the blood has been found to be uniformly infective during the so-called latent periods, when no trypanosomes can be found in the blood by microscopic examination.

II. *Extrusion of Granule.*

The original observations have been repeatedly verified during the past 18 months, and we have been able to satisfy ourselves completely that extrusion of granules is a constant feature of trypanosomal infections.

The phenomenon has been observed in all species of trypanosomes studied with the exception of *T. lewisi.*   We were able to assure ourselves of the presence of granules in that trypanosome, but the movements are so active that definite extrusion was never witnessed by either of us.   On account of the high degree of motility the species was unsuitable for work on this subject, and prolonged observations were not made.   The mechanism of extrusion has been studied in detail in *T. nanum* and *T. gambiense.*

(1) *T. nanum.*—The strain was obtained from infected cattle from the White Nile district, and, for the purpose of these observations, was kept up by passage through gerbils.   This type of trypanosome is very convenient for the study of this process, as the granule is large and very evident and the trypanosome, whilst evincing active lashing movements, does not progress across the field of the microscope, but remains more or less stationary, so that there is no difficulty in watching the same trypanosome through all the phases over a period of several hours, if necessary.   Further, an animal can be selected at a period when extrusion is a frequent occurrence.

When extrusion is about to take place the granule begins to work its way slowly, but quite distinctly, from the centre of the trypanosome towards the pointed extremity.   Arrived there, it makes its way back to the centre. This takes place quite often—as many as seven or eight such movements having been observed.   During these passages the granule can be seen distinctly bulging the periplast as if becoming more and more superficial— this bulging being strikingly apparent at the pointed extremity.   Probably this movement is largely due to the movements of the trypanosome itself. Finally, the granule, stretching the periplast to a greater extent, is extruded suddenly from the pointed extremity and becomes a free element in the surrounding medium.   Plate 9, fig. 1, illustrates all these stages.

(2) *T. gambiense.*—Here the preparations were made direct from cases of human trypanosomiasis.

In this species the granules are multiple and move rapidly backwards and forwards in the long axis of the trypanosome.   They exhibit also a dancing

movement and appear to throw themselves against the periplast and rebound from it. Sometimes the granules approach the surface, and in so doing may actually cause a slight protrusion on the covering membrane. This seems to be preliminary to extrusion, as afterwards the granule may be shot out with a certain degree of force into the free fluid to some distance from the host. In this species extrusion is not as a rule effected from the extremity, but from some point near the middle of the trypanosome body.

In infections running a very rapid course—such as *T. brucei* and *T. rhodesiense* in white rats—extrusion is readily observed, whereas in sleeping sickness in man, a very chronic infection, prolonged search may be necessary. Certain intermediate types of the disease are particularly suitable for study of this subject—for instance, *T. brucei* in gerbils, as described above. In the course of this infection granules are not extruded when the trypanosomes first appear. At a later stage the phenomenon is easily seen, and again it cannot be observed just before disappearance of trypanosomes from the blood. These facts tend to confirm our opinion that extrusion occurs at a definite period in the life of an adult trypanosome.

Extrusion can be stimulated by the administration of drugs and by certain mechanical effects such as variations in osmotic conditions. Reference is made to extrusion induced by varying strengths of salt solution in a later section of this paper.

Under ordinary circumstances extrusion of granules does not appear to have a prejudicial effect on the trypanosome. In warm wet preparations it can be seen to continue its movements and it apparently lives as long as the others. On the other hand, it has been shown above that extrusion of granules, if occurring generally, apparently heralds a disappearance of trypanosomes from the blood and is, in fact, the precursor of a trypanolytic crisis. Under unfavourable circumstances, *e.g.* after treatment, extrusion is followed by rapid disintegration of the trypanosomes.

III. *Effect of Drug Treatment on Extrusion.*

Certain phenomena in connection with the liberation of granules have been observed after treatment with antimony. Cases of sleeping sickness were given an intravenous injection of metallic antimony, and gland-puncture wet preparations made at short intervals after treatment, 3 minutes, 5 minutes, and so on. These were examined by dark-ground illumination and the results of the observations are here described.

Extrusion of granules is more frequent. The exaggerated motility is one factor, and the protoplasm, and more particularly the periplast, seem to lose elasticity, with the result that the granules can get free more easily. If a

granule is forcibly ejected by energetic movements of the trypanosome it is flung out into the free fluid to some distance ; this is the most usual method.

Some trypanosomes seem to be acutely poisoned by the antimony, and death and complete dissolution occur very rapidly. This is more frequently seen when the preparation is made 5 to 7 minutes after injection of antimony. The trypanosome becomes anchored, its lashing movement slows down and comes to a standstill, the body swells and becomes bloated, losing its characteristic form. In this condition it is devoid of energy and can no longer forcibly extrude granules, but the latter have not suffered so severely and may still show an excited dancing movement inside the degenerate trypanosome body, which appears to give way before this activity, and the granule may ultimately work its way clear of the degenerate protoplasm and inelastic covering of the now dead, or dying, trypanosome.

In other instances the trypanosome does not die so rapidly and the granules, after continuing this dancing movement inside it for some time, gradually come to rest before the trypanosome has reached so advanced a state of degeneration as to permit a dancing granule to escape by its own efforts. The degeneration of the trypanosome continues till it has lost outline and refractility and can only be recognised as an ill-defined "ghost," enveloping the granules which are held in position—more or less in the original long axis of the trypanosome—by this viscid protoplasm. This is the last stage that can be seen in a dark-ground preparation where the objects are at rest. In the living subject, however, it is probable that this degenerate protoplasm would not be allowed to remain at rest, but would be broken up by the active currents and eddies and the granules would thus be set free.

Thus there are probably three methods by which a granule may be liberated from the parent trypanosome :—

(1) By the activity of the trypanosome—forcible extrusion.

(2) By the active movement of the granule in a rapidly degenerating trypanosome.

(3) By outside agencies, eddies, currents, etc., which may break up a degenerate trypanosome when the contained granules are unable to effect their escape.

In some cases extrusion occurs rapidly. A trypanosome has been seen to extrude two large granules and immediately afterwards break up—the whole process being complete in 20 minutes.

In the early preparations (3 minutes) the exaggerated motility is a prominent feature and forcible extrusion is most commonly seen ; in the later films (7 minutes) the antimony has had longer time to act and the

phase of hyperactivity has passed. It is then more usual to see the more gradual escape of the granule, and as the trypanosomes are "anchored" they can be kept under observation more easily. On several occasions where death has occurred slowly we have been able to watch a trypanosome for periods up to four hours. In the 20-minute preparations trypanosomes have never been found, but granules are very numerous. The activity of the freshly extruded granules after antimony is much greater than the movements of granules seen before treatment.

IV. *The Free Granule.*

The granule free in the blood or fluids is seen to be a small spherical or pear-shaped body. In dark-ground preparations it is seen to be highly refractile, and by its activity it causes considerable disturbance in the surrounding fluid; with vital staining this young granule takes on the stain rapidly and uniformly, and seems to be undifferentiated. It frequently remains near its former host for some little time before showing independent movement. At first only a dancing movement may be seen; this, however, is a preliminary phase, and soon the granule begins to move slowly across the field, turning over on itself. There is no doubt as to the motility: they have often been observed to move out of a microscope field in preparations where there was no question of currents, etc. In our opinion a pseudopodial protrusion appears early, which at first is short and rather thick.

In animal infections and in cases of sleeping sickness in man, granules are found in the blood, glands, and internal organs. They are, of course, much more numerous in animals in which the adult parasites appear in great numbers. In experimental animals granules have been found in the proximal glands 24 hours after inoculation. This fact seems to be of great importance.

The criterion in the recognition of granules must be motility,* but their greater affinity for such stains as toluidin blue is of undoubted assistance in distinguishing them from the countless small bodies seen in wet preparations, *e.g.* blood-platelets and leucocyte granules.

V. *Further Development of Granule.*

So far we have shown that the trypanosome discharges living elements endowed with motility, and showing the same reaction as nuclear material to toluidin blue. The further stages are more difficult to follow, as all stages

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* The addition of a small quantity of cherry-gum solution to the preparation will differentiate between Brownian and vital movement. It stops the former and slows the latter.—H. G. PLIMMER.

cannot be seen in any individual preparation. We have endeavoured, so far as possible, to correlate the various appearances met with ; at the same time we cannot be sure that we set them out in exact chronological order.

In the first place granules, like many other free bodies in the blood plasma, are liable to undergo phagocytosis, and have been seen in all conditions within polynuclear leucocytes.

Granules somewhat older have also been seen in hyaline mononuclear and in endothelial cells, but in cells of this type, on the other hand, the contained granules are quite unchanged, and we are unable to say that they are being destroyed. It is possible that they may be entering on an intra-cellular phase of existence. They have been very well seen by one of us (W. B. F.) in a large mononuclear leucocyte during examination of the blood of a cat infected with *T. nanum*. They have also been seen in endothelial cells in liver puncture preparations from cases of sleeping sickness.

The first change seen in the free granule is a slight enlargement and elongation, rendering it more definitely pear-shaped. Then one begins to note a slight differentiation of structure into a central area staining a dark blue or purple, and a peripheral zone which is only faintly tinted blue. The enlargement is progressive, and the body becomes more uniformly blue, while a small dark blue or purple spot is visible, varying in position from the centre of the body to the apex. This may be assumed to be the earliest differentiation of cytoplasm from nuclear material. At this stage there is sometimes a definite flagellum-like projection which is usually short and rather thick, and more like a pseudopodium (Plate 11, fig. 2).

The same early forms have been studied in dark-ground preparations from bone-marrow in animals infected with *T. nanum* and are illustrated in Plate 9, fig. 2, A to I.

From this point the body enlarges, and the flagellum-like body becomes relatively, if not actually, reduced in size, so that forms are seen as in Plate 11, figs. 3 and 4. Later on the mass of chromatic material divides, and two are seen—one much smaller than the other. The body then becomes more rounded. Some are regularly spherical, while others show projections from various points, and have on surface view a roughly triangular appearance.

At this time of their development they resemble very closely the Leishman-Donovan bodies in Kala-azar ; they are found sometimes in enormous numbers in lungs, bone-marrow and spleen. Death in acute trypanosomiasis is caused by plugging of the cerebral capillaries with these forms. This cause of death is very similar to that in pernicious malaria.

From this stage—the binucleate body—there appear to be two directions

in which the further development may proceed. The body may enlarge slightly, develop a true flagellum from the neighbourhood of the micro-nucleus, and then become longer. This increase in length continues, and the macro- and micro-nucleus in this process become further separated; the flagellum comes to lie along the margin, and this form can now be recognised as an early immature trypanosome. There is no undulating membrane, but development proceeds till the adult form is reached.

On the other hand, the circular form may enlarge to a greater degree, and show a larger amount of a pale-blue staining cytoplasm that seems characteristic of young forms. The nucleus and micronucleus then undergo division by schizogony, but remain within the single mass of cytoplasm. The time of appearance of the flagellum seems to be variable, but ultimately all the pairs of macro- or micro-nuclei come to have a flagellum with a fan-shaped origin usually projecting beyond the margin of the cytoplasm.

Plate 11 shows forms with two, four, and eight macro- and micro-nuclei and flagella. We have seen indications of similar forms in vital pre-parations, but the latter cannot show the same detail as fixed and stained preparations. We have no knowledge as to the conditions which determine either of these events—possibly in the latter case there may be some sexual process either in the cells or fluids.

Many of these bodies are identical with the Plimmer and Bradford bodies, which they described in 1902,[*] and we have found them in preparations made from many different animals and from man, of glands, internal organs and bone-marrow. They show when living undoubted motility, but the early granule shows much more active movements than these later forms. The fact of their showing this vital property, however, precludes any possibility of their being degeneration forms.

In a few cases of sleeping sickness in man some other bodies have been seen by the vital method in fluid obtained by liver puncture. In the majority of instances some blood was mixed with the liver juice; this diluted the fluid and the bodies were very scanty, but the appearances presented suggested that some process of division was going on. Protoplasmic masses were seen containing four or eight small ovoid bodies taking on nuclear stain, but there was no nuclear differentiation. These were seen only in wet preparations, and could not be preserved.

Another form was seen as a fusiform body lying round a segment of the periphery, apparently of a mononuclear cell. It suggested an immature trypanosome, and this idea was confirmed by the presence of similar bodies free in the liver juice showing slight sluggish movement.

[*] 'Quart. Journ. Micros. Sci.,' February, 1902.

VI. *Fixed and Stained Specimens.*

The foregoing sections have dealt with living trypanosomes, but we were not able by the ordinary methods to make permanent preparations showing the various stages and forms, and demonstrating the staining reactions of the granule from its origin as a nuclear bud onwards.

Mr. H. G. Plimmer, F.R.S., has appended a note describing special fixing and staining methods devised by him, and we wish to state that it is only by the use of these methods that we have been able to confirm the appearances we have described in unfixed wet preparations, together with the differentiation between vital and nutritive granules.

In regard to the granule within the trypanosome, films have been stained showing the granule taking origin from the macro-nucleus itself as a small bud with characteristic chromatin reaction. All the stages of separation have been seen till the granule is a small, independent, dense, chromatin-staining mass in the cytoplasm (Plate 10, figs. 1–6). The granules, as stated, vary in number, and are most frequently seen between the macro- and micronucleus. They stain a deep red and show a remarkable contrast to the food granules which have taken on the iodine reaction from the fixation, and are visible as bluish-staining bodies or sometimes as a fused mass. This can be better seen in certain bird trypanosomes on account of their large size.

In a certain number—probably the larger number—of instances the granule at some stage of its development is surrounded by a faint-staining hyaline circular or ovoid area. It is probable that in such cases the granule is really within a vacuole (Plate 10, fig. 8). Sometimes the granule appears to be spherical, but in other cases, even when being budded off from the nucleus, it already shows as an elongated, pear-shaped body; this is well seen in Plate 10, figs. 2–4.

Granules can be seen actually causing a protuberance on the periplast and evidently on the point of being extruded. Others have been fixed when half-way out, while free granules, which have just effected their escape, have been seen lying close to the parent trypanosome. The early free granule takes on the chromatin stain deeply, and is identical with the body observed by the vital method.

The observations as to phagocytosis have been confirmed and more advanced forms, showing a macronucleus, micronucleus, and flagellum, have also been seen within polynuclear cells, and rounded forms resembling the Leishman-Donovan body have been demonstrated in large mono-nuclear cells.

All stages have been seen from the early free granule; the protoplasm

becomes more visible, and increases in amount; the nuclear material becomes differentiated from it and more concentrated, and then we are able to see early forms with a macronucleus and micronucleus. The macronucleus in the circular forms may be spherical or may become elongated and spread out along the periphery. Some forms show much more protoplasm : it stains a pale blue and sometimes shows some faint pink granules. The flagellum varies in length, but is relatively much longer than that of the adult trypanosome. In the internal organs, and especially in the lung, there may be enormous numbers of these small rounded bodies with macro- and micro-nuclei, with or without flagella, sometimes separate and sometimes massed together.

A further stage has been observed in these masses; they have been seen just on the point of disruption, some of the small bodies were separating, and lay at varying distances from the main mass. Each showed the two nuclear elements with a small body of homogeneous cytoplasm.

In addition, forms such as mentioned on p. 385 have been seen—large masses of protoplasm with two, four, or eight macronuclei, and corresponding micronuclei, which are, as a rule, placed close to the macronuclei. and stain very densely. The flagellum can be seen arising from a line equal in length and close to the micronucleus, in a fan-shaped collection of very fine filaments which unite to form a flagellum (Plate 11).

In smears of blood or organs advanced single forms—*i.e.* with one macro-nucleus, micronucleus and flagellum, and a relatively large amount of protoplasm—can be seen, and all stages from this to the adult trypanosome (Plate 11). A series has been prepared showing an almost imperceptible gradation from the granule stage up to adult trypanosomes.

Up to this point we have only referred to the work of Bradford and Plimmer in their paper on *Trypanosoma brucei* and its development. In this paper and in the plates they have described and figured the granules within the trypanosomes, the free early bodies, the more advanced single forms called "amœboid" and the disrupted schizogonous bodies called "plasmodial masses."

Our work was carried out at a time when we had no access to the paper, and this makes it all the more remarkable that the forms we describe should so closely resemble, and indeed confirm, many of the appearances described in 1902, and we feel that in many respects we can add little to the original work, beyond demonstrating the vital properties of these bodies.

We should like to draw attention to the fact that early granules, forms with short flagella and small round forms, are figured by Mott* in his

* 'Reports of the Sleeping Sickness Commission of the Royal Society,' No. VII December, 1906.

"Histological Observations on Sleeping Sickness and other Trypanosome Infections."

VII. *Some Animal Experimental Work in Reference to Granules.*

A number of experiments were undertaken to ascertain if it were possible to infect animals by granules alone.   To do this, fluid containing granules and no trypanosomes was required.   It was thought possible that the granules (if reproductive elements) might prove more resistant to changes in their environment than adult trypanosomes.   In order to test this, blood showing a heavy infection was added to a hypertonic salt solution, up to 2 per cent.

It was found on mixing one volume of infected blood with two to three of salt solution and keeping it at temperatures between 34° and 38° C., that after standing for 5 to 10 minutes individual trypanosomes began to swell up and become globular and the contained granule or granules to become active, moving about in the now spherical trypanosomes ; after a short period the granules escaped from the containing membrane and became free.   The remnant of the trypanosome was left as a faintly discernible spherical body with no characteristic features.

This process of escape of granules continued until no formed trypanosomes could be found; at the end of from half to three-quarters of an hour the process, as a rule, appeared complete.   There are apparently several factors which influence the occurrence of this phenomenon, the temperature, the hypertonicity of the solution, the stage of development of the trypanosomes, and the strain worked with.

If, whilst looking at one of these slides during the process, an individual trypanosome be watched, it will be noticed that its active movements suddenly become slowed, and then, as though blown steadily out by some entering fluid, the trypanosome, in the course of about 3 to 10 secs., is changed from its usual shape to that of a round body in which the granule or granules are freely motile.   The escape of the granule takes place, as a rule, a few minutes after this.

Infection was obtained repeatedly, and the following are details of two positive results :—

No. I.

November 5.—Gerbil (F. 10) injected with about 0·2 c.c. of treated blood obtained from a gerbil infected with *T. nanum* (heavy infection).   The injected blood was treated with sodium chloride solution 2 per cent. and sodium citrate 1 per cent. for one hour. At the moment of injection no living trypanosomes could be distinguished ; sphere forms and free granules very numerous.

November 10.—Trypanosomes first found in blood.

November 11.—Trypanosomes very numerous.

November 14.—Gerbil found dead ; spleen very large.

<div align="center">No. II.</div>

November 5.—Gerbil (F. 11) injected with blood (0·2 c.c.) obtained as above, but after two hours' standing no trypanosome could be seen, only round forms and free granules.

November 12.—Trypanosomes first found in blood.

November 14.—Trypanosomes very numerous.

November 15.—Gerbil found dead ; spleen very large.

The average time of infection in gerbils is four to six days after ordinary inoculation. Similar results were also obtained with dogs.

These experiments are, of course, not absolutely conclusive, but so far as could be ascertained microscopically the granules were the only discernible remnants of the trypanosomes which retained their characteristic form.

Further experiments were also made to trace if possible the fate of granules so injected into animals. Inoculations were made with solutions containing large number of free granules, and the animals were killed before trypanosomes could be found in the blood. Granules and the later forms in various stages of development were found in the proximal glands, also in the internal organs.

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Note on a New Method of Blood Fixation.

By H. G. PLIMMER, F.R.S.

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During some years of work on the blood of animals, many methods of fixation have been tried, principally with the view of obtaining a better fixation of blood parasites. The method described below has fulfilled this object better than any other, and is more faithful than even osmic acid.

The use of iodine for the fixation of unicellular organisms dates from the work of Kent in 1881 on the Infusoria, but the application of it to blood is, so far as I know, new.

I have used iodine in two forms, in vapour and in solution, and each has its special advantages. When a blood-film is exposed wet to the vapour from a solution of iodine in chloroform, the fixation of the various elements is practically instantaneous, as the penetrative power of iodine in this form is greater than that of any other fixative known to me ; there is less alteration both in form and size of the cellular elements and parasites than with any other fixative. When used in solution several things happen which are of value in enabling very fine structures to be more easily made out.

If blood be mixed with a solution of iodine in salt solution containing iodide of potassium, certain elements and parasites, especially trypanosomes, swell up so that the finer parts of their structure, for instance the nucleus and blepharoplast, are much clearer and more definite than with the ordinary